Sumoylation differentially regulates Sp1 to control cell differentiation

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The mammalian small ubiquitin-like modifiers (SUMOs) are actively involved in regulating differentiation of different cell types. However, the functional differences between SUMO isoforms and their mechanisms of action remain largely unknown. Using the ocular lens as a model system, we demonstrate that different SUMOs display distinct functions in regulating differentiation of epithelial cells into fiber cells. During lens differentiation, SUMO1 and SUMO2/3 displayed different expression, localization, and targets, suggesting differential functions. Indeed, overexpression of SUMO2/3, but not SUMO1, inhibited basic (b) FGF-induced cell differentiation. In contrast, knockdown of SUMO1, but not SUMO2/3, also inhibited bFGF action. Mechanistically, specificity protein 1 (Sp1), a major transcription factor that controls expression of lens-specific genes such as β-crystallins, was positively regulated by SUMO1 but negatively regulated by SUMO2. SUMO2 was found to inhibit Sp1 functions through several mechanisms: sumoylating it at K683 to attenuate DNA binding, and at K16 to increase its turnover. SUMO2 also interfered with the interaction between Sp1 and the coactivator, p300, and recruited a repressor, Sp3 to β-crystallin gene promoters, to negatively regulate their expression. Thus, stable SUMO1, but diminishing SUMO2/3, during lens development is necessary for normal lens differentiation. In support of this conclusion, SUMO1 and Sp1 formed complexes during early and later stages of lens development. In contrast, an interaction between SUMO2/3 and Sp1 was detected only during the initial lens vesicle stage. Together, our results establish distinct roles of different SUMO isoforms and demonstrate for the first time, to our knowledge, that Sp1 acts as a major transcription factor target for SUMO control of cell differentiation.

transcription regulation | eye development | crystallin gene expression

The conjugation of small ubiquitin-like modifiers (SUMOs) to protein substrates (named sumoylation) is a critical post-translational modification with diverse cellular functions (1). Three major SUMO isoforms (SUMO1, -2, and -3) were identified in vertebrates. Although the mature SUMO2 and SUMO3 share a very high level of sequence identity (97%) and cannot be immunologically discriminated (thus referred to as SUMO2/3), they significantly differ from SUMO1, with only 45% identity (2, 3). Recent studies using proteomics revealed that SUMO1 and SUMO2/3 can be targeted to both distinct and overlapping sets of substrates (4). However, whether SUMO1 and SUMO2/3 have redundant or different functions in vivo is not clear because inconsistent results have been reported in SUMO1 knockout mice (5, 6).

SUMO conjugation is executed by three enzymes. The activating enzyme E1, a heterodimer of SAE1 and SAE2, transfers SUMO to the single E2-conjugating enzyme Ubc9, which either sumoylates the substrate alone, or cofunctions with different E3 ligases. Sumoylation is highly dynamic and can be rapidly reverted by sentrin-specific proteases (SENPs) (7). Functionally, it regulates many cellular processes, including cell differentiation (8–11). In ocular tissues, sumoylation helps to determine the differentiation of cone versus rod photoreceptors (11). Our recent study revealed that SUMO1-mediated sumoylation is an indispensable step toward activation of p32 Pax-6, a master regulator of eye and brain development (12). Although the effects of sumoylation on individual targets in regulating cell differentiation and other biological processes are being unraveled, it remains largely unknown how SUMO isoforms regulate cell differentiation and whether SUMO1 and SUMO2/3 display distinct functions.

The vertebrate lens is an attractive model for studying cell differentiation (13). In this tissue, cell proliferation and differentiation occur throughout life. Differentiation of the vertebrate lens starts from the lens vesicle (LV) stage. Cells in the anterior of the LV retain epithelial morphology and proliferative capacity whereas the posterior cells elongate and differentiate into primary lens fiber cells (LFCs). The lens constantly grows throughout life via continued proliferation and differentiation of lens epithelial cells (LECs) in the germinal zone into secondary LFCs. Formation of both primary and secondary LFCs is characterized by elongated cell shape, accumulation of differentiation-specific proteins such as β-crystallins, and loss of subcellular organelles.

Significance

The mammalian small ubiquitin-like modifiers (SUMOs) are actively involved in regulating differentiation of different cell types. However, the exact functions of SUMO1 and SUMO2/3 have not been defined. Here, we demonstrate for the first time, to our knowledge, that SUMO1 promotes cell differentiation whereas SUMO2/3 inhibits cell differentiation. Mechanistically, we demonstrate that specificity protein 1 is the major target activated by SUMO1 conjugation but is repressed by SUMO2/3-mediated sumoylation via our newly identified K683 residue, as well as the known K16 site.

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In vitro and in vivo, lens differentiation can be triggered by extracellular growth factors, such as basic fibroblast growth factor (bFGF) (14). When cultured in high concentration of bFGF (50~100 ng/mL), LECs elongate, migrate, and become multilayered to form LFCs (13, 14). Furthermore, accumulation of differentiation-specific β-crystallin is induced upon prolonged culture (14). However, loss of nuclei was not detected in this condition (14), implying that the in vitro model mimics early stages of in vivo fiber differentiation.

Using this system, we explored the functions of SUMO1 and SUMO2/3 in cell differentiation. Our results reveal that SUMO1 and SUMO2/3 differ significantly in abundance, localization, and substrate targets. Although SUMO1 promotes cell differentiation, SUMO2/3 inhibits this process. Such distinct functional differences are in part mediated through transcription factor Sp1. Although SUMO1 directly sumoylates Sp1 to positively regulate β-crystallin genes, SUMO2 inhibits Sp1 function by attenuating its DNA binding activity, decreasing its protein stability, and suppressing its interactions with the coactivator p300. Together, our results demonstrate distinct functional roles and mechanisms of action for SUMO1 and SUMO2/3 in regulating lens cell differentiation.

Results

SUMO1 and SUMO2/3 Display Significant Differences in Expression and Cellular Localization During Mouse-Lens Development. To explore the possible roles of SUMO1 and SUMO2/3 in lens-cell differentiation, we examined their expression and localization patterns from embryonic day 9.5 (ED9.5) to day 3 lens using immunohistochemistry (IHC). As shown in Fig. S1, strong signals of SUMO1 and SUMO2/3 were ubiquitously detected in all LV cells (Fig. S1) (ED11.5), and a similar pattern of SUMO1 and SUMO2/3 expression was detected at ED13.5 (Fig. S1). At ED16.5, when active fiber differentiation takes place, homogenous SUMO1 distribution was detected in nuclei of LECs at the anterior portion of the lens (Fig. 1 *A*, *b* and *b2*). At the transition zone, SUMO1 labeling started to move toward the nuclear periphery (NP) (Fig. 1 *A*, *b* and *b1*). In elongating LFCs moving out of the transition zone, SUMO1 labeling was primarily detected at the NP (Fig. 1 *A*, *b* and *b1*). For LFCs localized at deeper layers undergoing terminal differentiation, the NP localization of SUMO1 was more prominent (Fig. 1 *A*, *b* and *b2*). By contrast, SUMO2/3 staining was prominently represented by intensity change during lens differentiation. Strong SUMO2/3 labeling was detected in LEC nuclei, with a decreased signal in elongating primary LFCs (Fig. 1 *A*, *d* and *d1*), and eventually the signal disappeared in terminally differentiated LFCs (Fig. 1 *A*, *d* and *d2*). Similar patterns were observed in the day 3 lens (Fig. S1). In the adult lens, SUMO1 labeling was mainly seen in LECs, with a detectable level in the differentiating LFCs of the transition zone (Fig. S1). In contrast, the SUMO2/3 signal was much lower in the LECs and became hardly detectable in LFCs of the transition zone (Fig. S1).

SUMO1 and SUMO2/3 Are Conjugated to Different Targets During Mouse-Lens Differentiation. The different distributions of SUMO1 and SUMO2/3 in differentiating LFCs suggest that they may be conjugated to different targets. Indeed, Western-blot analysis (WB) suggested that SUMO1 and SUMO2/3 modified distinct targets (Fig. 1*B*). Neither SUMO1 nor SUMO2/3 was detected in their free forms (in Fig. 1*B*, no bands with 11 kDa were observed), indicating that the observed IHC signals (Fig. 1*A* and Fig. S1) represent sumoylated proteins. Interestingly, an increased SUMO1 modification of several lower molecular weight proteins was observed in adult lens (Fig.1*B*, red arrow heads). The older lens has a higher content of secondary LFCs than embryonic or younger lenses, and WB conducted on different lens compartments showed that the newly SUMO1-modified

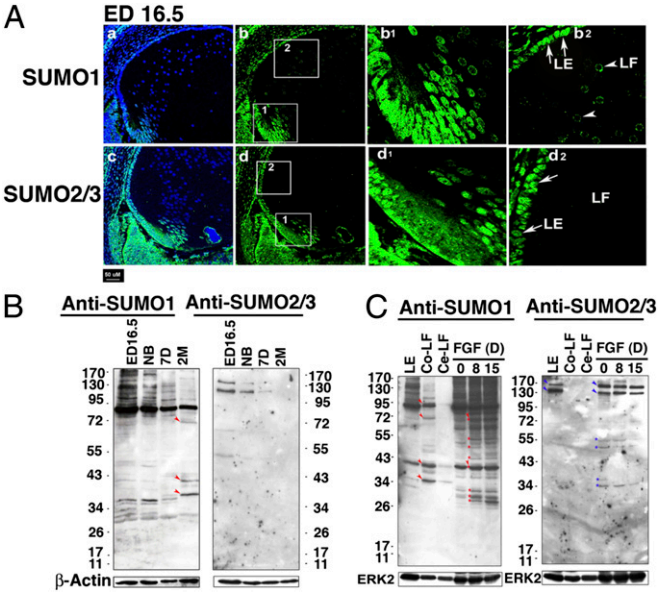

Fig. 1. SUMO1 and SUMO2/3 are differentially expressed and conjugated during lens differentiation. (*A*) Cryosections of mouse eye at embryonic day (ED) 16.5 were processed for IHC and observed under a confocal microscope. Lens epithelial (LE) cells and lens fiber (LF) cells are indicated by arrow and arrow head, respectively. (*a* and *c*) Overlapping of SUMO1 or SUMO2 (green) image with nuclei (DAPI staining, blue). (*b* and *d*) IHC staining of SUMO1 or SUMO2. (Magnification: *a*–*d*, 100×.) (*b1*, *b2*, *d1*, and *d2*) Magnifications of boxed areas in *b* and *d*, respectively. (*B*) WB showing conjugation of SUMO1 (*Left*) and SUMO2/3 (*Right*) in mouse lenses of indicated ages. NB, newborn; 7D, 7 d; 2M, 2 mo. The newly sumoylated protein species in the adult lens are indicated by red arrow head. (*C*) WB showing SUMO1 (*Left*) and SUMO2 (*Right*) in adult mouse lenses and bFGF-induced αTN4-1 cells. Protein species with enhanced SUMO1 modification or decreased SUMO2 modification under bFGF treatment were indicated by red asterisk or blue asterisk, respectively. Those also modified by SUMO1 or SUMO2 in vivo were labeled with red or blue arrow head, respectively.

species indeed came from the secondary LFCs (the Co-LF lane of Fig. 1*C*, *Left*). A comparison of the WB result (Fig. 1*B*, *Left*) with the Coomassie blue staining (Fig. S2*A*) or silver staining (Fig. S2*B*) of the same samples (indicated by black or white arrow heads, respectively) suggested that the increased SUMO1 signals appeared to come from enhanced sumoylation instead of altered expression of SUMO1 substrates. By contrast, SUMO2/3 modifications were limited to the same substrates but showed a significant reduction in density as lens ages (Fig. 1*B, Right*). Furthermore, SUMO2/3 conjugates were detected only in LECs of adult mice (Fig. 1*C*, *Right* and Fig. S1). Reduced SUMO2/3 conjugation is likely derived from decreased SUMO2/3 expression (Fig. S2*D*) rather than enhanced desumoylation because no free SUMO2/3 were detected (Fig. 1*C*, *Left*). In addition, dot blot-based WB (Fig. S2 *C* and *D*) showed that SUMO1 is more abundant than SUMO2 and is thus the major SUMO isoform in the lens. Decreased SUMO2/3 expression is necessary for normal lens differentiation to occur (see the section below).

SUMO1 Promotes, Whereas SUMO2/3 Inhibits, Lens-Fiber Differentiation. The differential expression profiles, cellular localization, and conjugation patterns of SUMO isoforms imply that SUMO1 and SUMO2/3 may play distinct roles in lens differentiation. To explore this possibility, mouse lens epithelial cells (MLECs) were subjected to bFGF treatment to induce fiber differentiation (Fig. S3). Although bFGF enhanced SUMO1 conjugation, it modestly decreased SUMO2 modification (FGF lanes of Fig. 1*C*). To analyze the functions of SUMO isoforms in bFGF-induced LFC

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differentiation, we stably expressed SUMO1, SUMO2, or SUMO3 in MLECs (αTN4-1 line); stable clones were established and expression of SUMO1/2/3 was confirmed (Fig. S4*B*). The stable clones were treated with or without 100 ng/mL bFGF to induce fiber differentiation. After bFGF treatment, both vector GFP- and SUMO1-transfected αTN4-1 cells demonstrated multilayered LFC morphology with lentoid bodies (Fig. 2 *A*, *a′*–*c′* and *d′*–*f′*). In contrast, SUMO2- and SUMO3-transfected αTN4-1 cells still remained as a monolayer after 15-d bFGF treatment (Fig. 2 *A*, *g′*–*i′* and *j′*–*l′*). Consistent with these morphological differences, bFGF-induced β-crystallin was suppressed in both SUMO2- and SUMO3-transfected αTN4-1 cells (Fig. 2*B*). To confirm that SUMO1 is necessary for lens differentiation, SUMO1 was stably silenced in αTN4-1 cells (Fig. S4*C*). As a result, bFGF-induced fiber differentiation was significantly inhibited by SUMO1 knockdown (Fig. 2 *C* and *D*) but not by SUMO2 or SUMO2/3 knockdown (Fig. 2 *C* and *D*). The observed suppression of differentiation correlated with enhanced cell proliferation of SUMO1-silenced cells (Fig. 2*E*). Together, our results reveal that SUMO1 promotes, but SUMO2 and -3 inhibit bFGF-induced LFC differentiation.

SUMO1 and SUMO2/3 Differentially Regulate Sp1 Activity. To explore the possible molecular mechanisms mediating the regulation of lens differentiation by SUMO1 and SUMO2/3, we explored their targets. Knowing that, during lens differentiation, β-crystallins are greatly induced, we examined their core promoters and found that the most common consensus cis-element is the site bound by the Sp1/Sp3 transcription factors (Fig. S5 *A*–*C*) (15). Electrophoretic mobility-shift assay (EMSA) confirmed that Sp1 and Sp3 directly bound to the core promoter regions of human βB1-, βB2-, and βB3-crystallins (Fig. S5 *D*–*F*). Furthermore, Sp1 significantly transactivated the β-crystallin gene promoter-driven luciferase reporter constructs (Fig. S5 *G*, *a*–*c*) and endogenous β-crystallin expression (Fig. S5 *G*, *d*–*f*). This Sp1-mediated activation disappeared when the Sp1 binding sites were mutated in the reporter-gene constructs (Fig. S5 *G*, *a*–*c*).

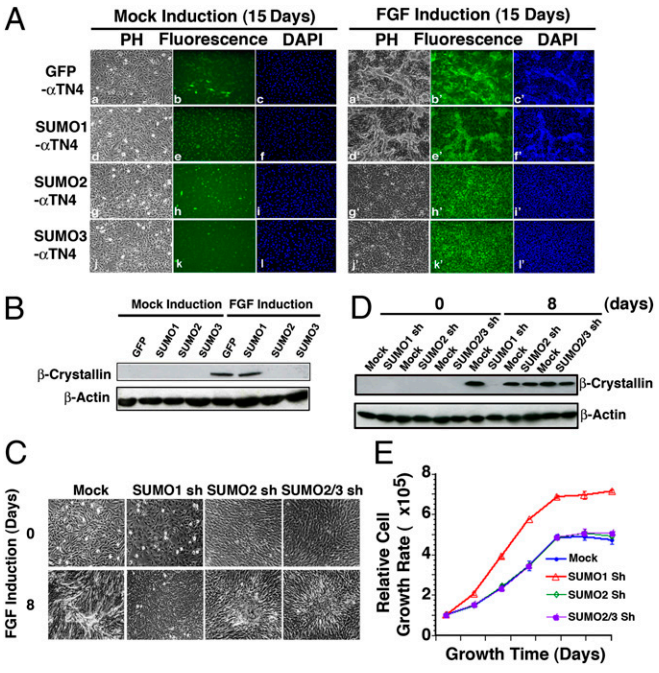

Fig. 2. Contrasting effects of SUMO1 and SUMO2/3 on bFGF-induced fiber differentiation. (*A*) αTN4-1 cells stably expressing GFP, GFP-SUMO1, GFP-SUMO2, or GFP-SUMO3 were left untreated (*a*–*l*) or treated with bFGF for 15 d (*a′*–*l′*). Cell morphology was observed under a phase contrast microscope (PH) or under a fluorescence microscope to detect GFP or nuclei (DAPI). (Magnification: 50×.) (*B*) WB to show β-crystallin in four stable cell lines without (Mock; 15 d) or with (FGF; 15 d) bFGF induction. (*C*) Established mock (Mock), SUMO1 knockdown (SUMO1 sh), SUMO2 knockdown (SUMO2 sh), or SUMO2/3 knockdown (SUMO2/3 sh) αTN4-1 cells were treated with bFGF to induce fiber differentiation for 8 d. (Magnification: 50×.) (*D*). WB to show β-crystallin expression in Mock, SUMO1 sh, SUMO2 sh, and SUMO2/3 sh cells during bFGF-induced fiber differentiation. (*E*) MTT assay to evaluate the cell proliferation in different cells indicated. Experiments were done in triplicate and represent mean values ± SD.

Next, we explored whether SUMO1 and SUMO2/3 can modulate Sp1 activity. Coexpression of Sp1 with SUMO1 enhanced luciferase reporter gene activity (Fig S5 *G*, *a*–*c*) ($P < 0.05$ in all cases) and endogenous β-crystallin expression (Fig. S5 *G*, *d*–*f*). In contrast, coexpression of Sp1 with SUMO2 significantly down-regulated reporter-gene activity and endogenous β-crystallin expression (Fig. S5*G*). As control, expression of SUMO1 or SUMO2 alone did not yield obvious changes in luciferase activities and endogenous β-crystallin expression (Fig. S5*G*). Thus, SUMO1 and SUMO2 differentially regulate Sp1 transcriptional activity in the lens system.

Conjugation of Sp1 by SUMO1 and SUMO2 at K683 Has Differential Effects on DNA Binding Activity. To understand how SUMO1 and SUMO2 differentially affect Sp1 activity, we examined their effects on Sp1 DNA binding. Previous studies have revealed that Sp1 has a conserved SUMO acceptor site at K16 (16). A careful examination of the C-terminal portion of the Sp1 primary structure identified another putative sumoylation site at K683 (Fig. S6 *A*, *a*). In vitro-translated Sp1 DNA binding domain (DBD) containing this site was subjected to sumoylation by SUMO1 or SUMO2. Sp1 K683 was preferentially sumoylated by SUMO2 (Fig. S6 *A*, *b*), and SUMO2-conjugated Sp1 DBD displayed weaker binding to the βB1-crystallin gene promoter than SUMO1-conjugated Sp1 DBD did (compare lane 6 with lane 8 in Fig. S6 *B*, *b*). When K683 was mutated to arginine (R), the difference in the DNA binding patterns by Sp1 DBD after SUMO1 or SUMO2 conjugation disappeared (compared lane 12 with lane 14 in Fig. S6 *B*, *b*). Thus, differential conjugation of Sp1 by SUMO1 and SUMO2 at K683 led to differential effects on Sp1 DNA binding activity.

SUMO1 and SUMO2 Exert Opposing Effects on Sp1 Stability. We next examined Sp1 stability in response to conjugation with SUMO1 or SUMO2. Sp1 protein expression was examined in nuclear (NE) and cytoplasmic fraction (Cyto) extracted from mock or bFGF-induced cells transfected with vector, SUMO1, or SUMO2 (Fig. 3*A*). bFGF treatment increased nuclear Sp1 level in both GFP- and SUMO1-transfected αTN4-1 cells, but Sp1 expression was reduced in SUMO2-transfected αTN4-1 cells either before or after bFGF treatment, compared with the vector-transfected cells under the same conditions (Fig. 3 *A*, *a*). Consistently, cotransfection with SUMO2 into human lens epithelial (HLE) cells also decreased Sp1 expression (Fig. 3 *A*, *b*). This SUMO2-mediated Sp1 decrease is not derived from decreased mRNA level (Fig. 3 *A*, *c*), but from the decreased protein stability as revealed with cyclohexamide treatment (to block protein synthesis, Fig. 3 *A*, *d*). In contrast, cotransfection with SUMO1 significantly increased the Sp1 protein expression (Fig. 3 *A*, *d*). This result is interesting because a previous study with cancer cells showed that modification by SUMO1 at K16 decreased Sp1 stability (16). To confirm the above results, we mutated K16 into R in Sp1 and cotransfected Sp1-K16R with SUMO1 or SUMO2. Mutation of K16 did not affect SUMO1-induced Sp1 increase but suppressed SUMO2-dependent Sp1 degradation (Fig. 3 *A*, *d*). Thus, SUMO1-enhanced Sp1 expression may be derived from enhanced mRNA translation (17) but is not due to direct modification of Sp1. Taken together, SUMO1 and SUMO2 have differential effects on Sp1 expression.

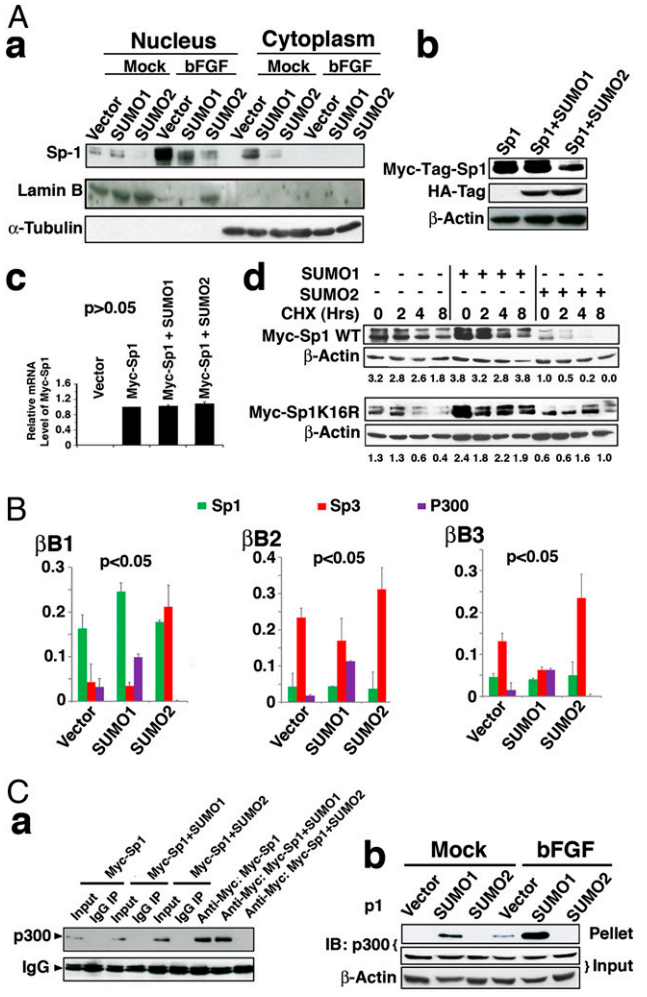

Fig. 3. SUMO1 and SUMO2 differentially regulate Sp1 stability and interactions with other factors. (*A*) SUMO2 overexpression decreased Sp1 stability. (*a*) WB to show Sp1 level in nucleus and cytoplasm of the indicated cell lines without or with bFGF treatment. (*b*) Myc-tagged Sp1 (Myc-Tag-Sp1) was cotransfected with equal amount of HA-tagged SUMO1 or -SUMO2 into HLE cells. (*c*) mRNA level of Sp1 was examined by qPCR at the indicated transfections. (*d*) Cycloheximide (100 μg/mL) treatment to assess the effect of SUMO1 or SUMO2 on the steady level of Sp1 wild type (WT) or K16R mutant (K16R) in HLE cells. The numbers below the β-Actin lanes are the relative levels of Sp1 expression under different conditions. (*B*). qChIP showing that SUMO2 overexpression enhances Sp3 but suppresses p300 binding into βB1, βB2, and βB3 promoters. (*C*) SUMO2 overexpression inhibits interactions between Sp1 and p300. (*a*) Myc-Sp1 was cotransfected with SUMO1 or SUOM2 and then precipitated by Myc-tagged magnetic bead conjugate. Interactions with p300 under indicated transfections were detected by WB using anti-p300 and anti-IgG antibodies (an equal amount of normal IgG was also added into input samples for loading comparison). (*b*) SUMO2 overexpression inhibits interactions between Sp1 and p300 during bFGF induction. GFP, SUMO1, and SUMO2-αTN4-1 cells were untreated (Mock) or treated with 100 ng/mL bFGF to induce fiber differentiation. A total of 5 mg of proteins were used in each IP. The resulting precipitates were separated by 6% SDS/PAGE gel and analyzed by WB.

SUMO1 Promotes, but SUMO2/3 Suppresses, the Interactions Between Sp1 and p300. Besides the effects on DNA binding and stability, SUMO2 may suppress the transcriptional activity of Sp1 by recruiting a repressor or preventing coactivator binding to β-crystallin promoters (18). Sp3 is known to act as a repressor of the Sp family (19), and our results showed that it could directly bind to the β-crystallin promoters (Fig. S5 *D–F*) and repress their activation by Sp1 (Fig. S6 *C*, *a–c*). Histone acetyltransferase p300 is an important coactivator for Sp1 during cell differentiation (18). Quantitative chromatin immunoprecipitation (qChIP) showed that, although the Sp1 enrichment was not altered in each cell line, overexpression of SUMO2 led to a significantly higher Sp3 occupancy at β-crystallin promoters, and p300 enrichment was greatly suppressed (Fig. 3*B*). In contrast, higher p300 occupancy was found in SUMO1-αTN4 cells. We hypothesized that SUMO2 expression may interfere with the interaction between Sp1 and p300; thus, a coimmunoprecipitation (Co-IP)-linked WB was conducted. Indeed, cotransfection with SUMO2 led to decreased interaction between Sp1 and p300 (Fig. 3 *C*, *a*). Additionally, the endogenous Sp1 and p300 interaction was further investigated under bFGF treatment. Without the differentiation signal, interaction between endogenous Sp1 and p300 was detectable only in SUMO1-transfected cells. In the presence of bFGF, such interaction was induced in GFP-transfected cells and dramatically increased in SUMO1-transfected cells but was undetectable in the SUMO2-transfected αTN4-1 cells (Fig. 3 *C*, *b*). To further confirm the interference of SUMO2 conjugation with the interaction between p300 and Sp1, an in vitro direct binding assay was conducted. As shown in Fig. S6*D*, sumoylation of Sp1 with SUMO2 inhibited its interaction with p300, but sumoylation with SUMO1 enhanced the interaction.

Taken together, SUMO2 negatively regulated Sp1 activity by attenuating DNA binding, decreasing Sp1 protein stability, and interfering with the interaction between Sp1 and its coactivator p300.

To confirm the functional importance of sumoylation at K16 and K683 of Sp1 by SUMO1 and SUMO2/3, we conducted rescue study under Sp1 knockdown background with a shRNA plasmid targeting at the 3′-non translation region. Transfection of K16R mutant Sp1 into the Sp1 knockdown cells could not restore bFGF-induced differentiation (Fig. S7). In contrast, expression of the K683R mutant Sp1 rescued bFGF-induced differentiation (Fig. S7). Together, these results further confirm the importance of Sp1 sumoylation by SUMO1 and SUMO2 in regulating lens differentiation.

SUMO1 and SUMO2/3 Differentially Sumoylate Sp1 at Different Developmental Stages of Mouse Lens. To demonstrate that Sp1 is actually subjected to SUMO modulation in vivo, we first examined the possible colocalization of Sp1 and SUMO1, or Sp1 and SUMO2/3, in mouse embryonic lens. As shown in Fig. 4*A*, Sp1 and SUMO1 or SUMO2/3 can be colocalized in the lens vesicle as early as ED11.5. Notably, the colocalization of Sp1 and SUMO1 is relatively homogenous in the anterior LECs and posterior differentiating cells of the lens vesicle (Fig. 4*A*, *Upper*, arrow). In contrast, colocalization of Sp1 and SUMO2/3 is mainly seen in the anterior epithelium (arrow) but greatly reduced in the posterior cells undergoing differentiation into primary LFCs (Fig. 4*A*, *Lower*, arrow head). To confirm differential interactions between Sp1 and different SUMOs, Co-IP experiments were conducted. As shown in Fig. 4*B*, although Sp1 and SUMO1 formed a complex at both ED11.5 and newborn (NB) stages, a complex between Sp1 and SUMO2/3 was detected only at ED11.5 but not in the NB stage (Fig. 4 *B*, *a*), which mainly comprise differentiating lens fiber (LF) cells. Similar results were obtained when SUMO antibodies were used for the immunoprecipitation and when Sp1 antibody was used for immunoblotting (Fig. 4 *B*, *b*). To further confirm the differential regulation of the β-crystallin gene promoters by SUMO1 and SUMO2/3, we conducted qChIP assays. As shown in Fig. 4*C*, a sequential qChIP using anti-SUMO1 first and then anti-Sp1 antibodies revealed that SUMO1 and Sp1 were found bound to the β-crystallin gene promoters in the regions where the Sp1 binding sites were present. In contrast, binding by SUMO2/3 or SUMO2/3-conjugated Sp1 to these promoters in NB lens was at background levels because the sequential qChIP assays with

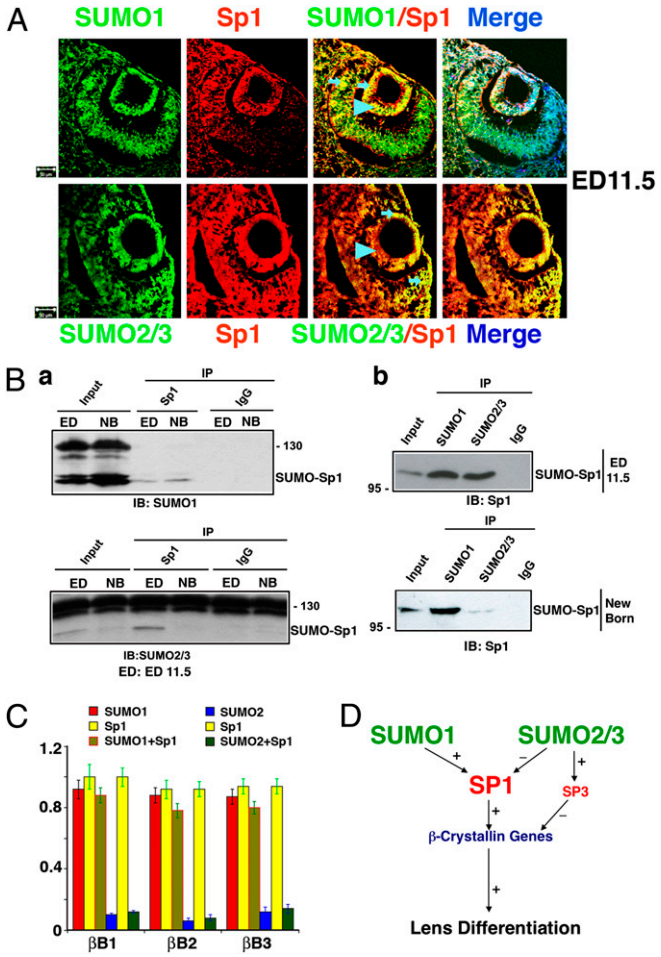

Fig. 4. SUMO1 and SUMO2/3 modulates Sp1 in mouse lens. (*A*, *Upper*) Colocalization (yellow) between Sp1 (red) and SUMO1 (green) was analyzed by IHC in ED11.5 mouse lens. (*Lower*) Colocalization (yellow) between Sp1 (red) and SUMO2/3 (green) was analyzed by IHC in ED11.5 mouse embryonic lens. The overlapping coefficiency for SUMO1 and Sp1 is 0.953 and is 0.921 for SUMO2/3 and Sp1 using the thresholds method (*SI Methods*). (Magnification: 50×.) (*B*) Co-IP to show modifications of Sp1 by SUMO1 or SUMO2/3. Total proteins were extracted from ED11.5 eye or NB mouse lens, immunoprecipitated by anti-Sp1 (*a*), anti-SUMO1, or anti-SUMO2/3 antibodies (*b*). The precipitated samples were immune-blotted with anti-SUMO1 (*a*, *Upper*) or anti-SUMO2/3 antibodies (*a*, *Lower*), or anti-Sp1 antibody (*b*). Note that both SUMO1 and SUMO2/3 were detected in proteins precipitated by anti-Sp1 antibody at ED11.5, but only SUMO1 detected at NB lens (*a*). Similarly, at ED11.5, SUMO-Sp1 signal was found in anti-SUMO1-and SUMO2/3-pelleted samples but not in control IgG (*b*, *Upper*). In NB lens, however, SUMO-Sp1 signal was found only in anti-SUMO1–pelleted sample but not in control IgG or anti-SUMO2/3–pelleted samples (*b*, *Lower*). (*C*) SUMO1 but not SUMO2/3 occupies βB1, βB2, and βB3 promoters as analyzed by qChIP. (*D*) Schematic diagram to show that SUMO1 and SUMO2 differentially regulate lens differentiation through Sp1. +, positive regulation; –, negative regulation.

anti-SUMO2 and anti-Sp1 yielded similar results with anti-SUMO2 alone. Taken together, SUMO1 and SUMO2/3 differentially control lens differentiation, and the transcription factor Sp1 acts as a key mediator during lens morphogenesis.

Discussion

Mechanisms for SUMO-Mediated Control of Cell Differentiation. Regulation of cell differentiation by SUMOs has been explored in numerous cases. In the retina, Pias3-mediated sumoylation of the transcription factor Nr2e3 converts it into a potent repressor of cone-specific gene expression, thus acting as a key mechanism for rod specification (11). Sumoylation dynamics also plays a key role in the reprogramming and differentiation of the human endometrial stromal cells (HESCs) (8). Global SUMO1 deconjugation occurred during HESC differentiation, correlating with altered E3 ligase and protease expressions (8). In contrast, during calcium-induced keratinocyte differentiation, overall enhanced SUMO1 modifications were accompanied by up-regulation of sumoylation system components (9). Although it is now well-established that sumoylation plays important roles in regulating cell differentiation (8–12), little is known regarding the impact of the individual SUMO paralogues. An early study has shown that haploinsufficiency of SUMO1 led to cleft lip and palate in embryonic mice (5), suggesting that SUMO2/3 do not substitute for SUMO1. However, more recent studies showed that SUMO1$^{+/-}$ and SUMO1$^{-/-}$ mice seemed to develop normally, suggesting that different SUMO isoforms may have redundant functions in vivo (6). At this stage, we do not know whether depletion of SUMO1 or SUMO2/3 will have prominent effects on lens development. Nevertheless, in the present study, several lines of evidence suggest that SUMO1 and SUMO2/3 have different functions. First, SUMO1 and SUMO2/3 showed clear difference in expression, cellular localization, and conjugation substrates. In differentiating LFCs, the SUMO1 signal was mainly detected at the nuclear periphery, and this nuclear-rim localization became prominent in LFCs undergoing terminal differentiation (Fig. 1*A*). By contrast, SUMO2/3 were detected only in undifferentiated LECs and a small portion of LFCs in the transition zone (Fig. 1 *A* and *C*). This confined localization of SUMO2/3 implies that SUMO2/3 either are not required for fiber differentiation or, more likely, have inhibitory functions on lens differentiation (see more discussion in the later part of this paragraph). Secondly, in a well established bFGF-induced LFC differentiation model (13, 14) (Fig. S3), globally enhanced SUMO1 conjugations were observed during this process, but SUMO2/3 modifications were nonresponsive to bFGF (Fig. 1*C*). Overexpression of exogenous SUMO1 and SUMO2/3 in MLECs resulted in different phenotypes. Similar to vector-transfected cells, overexpression of SUMO1 allows bFGF induction of lens differentiation (Fig. 2). In contrast, when SUMO2 and SUMO3 were overexpressed in MLECs, bFGF-induced fiber differentiation was markedly suppressed (Fig. 2). On the other hand, silencing of SUMO1 significantly inhibited bFGF-induced fiber differentiation and enhanced cell proliferation (Fig. 2), implying that expression of SUMO1 in differentiating fiber cells at the transition zone is necessary for cell-cycle exit and initiation of the fiber-differentiation program. In comparison, knockdown of SUMO2 or SUMO2/3 had little effect on bFGF-induced differentiation (Fig. 2). Thirdly, SUMO1 and SUMO2/3 differentially modulate Sp1 activity. Although SUMO1 enhances Sp1 activity, SUMO2/3 suppresses its activity, supporting the notion that down-regulation of SUMO2/3 expression is necessary for lens differentiation. Indeed, this result is exactly what we have observed in mouse lens (Fig. 1*B*). Thus, our results favor that SUMO1 and SUMO2/3 cannot substitute each other during lens development as observed in other tissues (5). The observed normal development with SUMO1 deletion reported in a more recent study (6) is likely derived from the situation that some mice with abnormal development under SUMO1 deficiency die at embryonic stage. Together, our present study reveals that SUMO1 and SUMO2/3 display distinct functions in controlling cell differentiation. Mechanistically, such functional difference is derived from their differential modulation of key transcription factors (see the section below).

SUMO1 and SUMO2 Differentially Modulate Sp1 to Mediate Cell Differentiation. To explore the molecular mechanisms by which SUMO1 and SUMO2 differentially regulate lens differentiation,

we explored their conjugated targets. We found here that the major lens differentiation marker genes coding for β-crystallins were expressed upon bFGF-induced differentiation in vitro or in vivo (Fig. 2). Moreover, we demonstrated that all of the β-crystallin gene promoters have well-conserved Sp1/Sp3 binding sites (Fig. S5 *A–C*). Sp1 is a universal transcription factor that has been shown to control expression of many genes from viral to cellular (15, 20). In the present study, the results of EMSA experiments demonstrated that Sp1 strongly binds to β-crystallin gene promoters (Fig. S5 *D–F*) and positively regulates their expression (Fig. S5*G*). Moreover, coexpression of the exogenous SUMO1 and SUMO2 with Sp1 differentially modulates these genes. Although SUMO1 enhanced β-crystallin gene expression, SUMO2 significantly inhibited these promoters (Fig. S5*G*). Furthermore, the Sp1 knockdown cells can only be rescued by a K683R (a SUMO2-favored sumoylation site) mutant but not by K16R mutant (Fig. S7).

At the molecular level, Sp1 conjugation by SUMO1 and SUMO2 has different outcomes. We found that Sp1 has a novel sumoylation site at K683 in the DNA binding domain, which shows preferred conjugation by SUMO2 (Fig. S6 *A*, *b*). SUMO2 conjugation at this site attenuates Sp1 DNA binding activity (Fig. S6*B*). Although both SUMO1 and SUMO2 can be conjugated to Sp1 at K16, conjugation of SUMO2 to this site significantly decreases Sp1 stability (Fig. 3*A*). In contrast, SUMO1 conjugation to Sp1 has no direct effect on protein stability (Fig. 3*A*). This result differs from a previous study with cancer cells where SUMO1 modification led to Sp1 degradation (16). Such an inconsistence may be due to cell-specific effects. Finally, SUMO1 and SUMO2 differentially direct Sp1 interactions with repressors and coactivators. Previous studies showed that the interaction with p300 enhanced Sp1 DNA binding activity (18). We found that coexpression of Sp1 with SUMO1 prevents its interactions with the sibling repressor, Sp3, but enhanced its interaction with the coactivator, p300 (Fig. 3*C* and Fig. S6*D*). In contrast, coexpression of Sp1 with SUMO2 led to the opposite effects: SUMO2 recruits Sp3 to the β-crystallin gene promoters (Fig. 3*B)* but prevents the association between Sp1 with p300 (Fig. 3*C* and Fig. S6*D*).

Because the outcomes of Sp1 conjugation by SUMO1 and SUMO2 are so different, progression of lens differentiation would require one of two conditions: either the lens must have a fine mechanism to ensure specific Sp1 sumoylation by SUMO1 vs. SUMO2/3 at specific developmental stages if they are expressed at similar levels, or, more simply, they display differential expression patterns such that SUMO1 is present when it is needed and SUMO2 disappears when it is not required. Our results demonstrate that the lens apparently adopts the latter strategy (Fig. 1 and Fig. S1). Although SUMO1 is strongly expressed and conjugated during the embryonic stage and is maintained at a certain level in the postnatal stages (Fig. 1 and Fig. S1), expression of SUMO2/3 is gradually decreased and is present only in the epithelial cells of the adult lens (Fig. 1*C* and Fig. S1). In this way, the presence of SUMO1 allows positive regulation of lens differentiation (Fig. 2 *A* and *B*). In contrast, the absence of SUMO2/3 in lens-fiber cells would pose no inhibition on lens differentiation. Moreover, the confinement of SUMO2/3 in LECs also ensured that LECs remain in the epithelial status. These observations would be consistent with results from both in vitro and in vivo studies. In the in vitro bFGF induction of lens differentiation, although SUMO1-conjugated proteins are gradually increased from day 0 to day 15 (Fig. 1*B*), the SUMO2/3-conjugated substrates are gradually decreasing during the same period. During normal lens development, Sp1 conjugation by SUMO1 was detected by homogenous localization in lens vesicle (ED11.5) (Fig. 4*A*) and by Co-IP in both ED11.5 and NB lenses (Fig. 4*B*). In contrast, Sp1 conjugation by SUMO2/3 was detected only by colocalization in the anterior epithelial cells of the lens vesicle (ED11.5) and was further confirmed by Co-IP (Fig. 4 *A* and *B*). At the NB lens, Co-IP confirmed that Sp1 was not modified by SUMO2/3 (Fig. 4*B*). Thus, our study here provides an important conclusion: down-regulation of SUMO2/3 as lens differentiation proceeds is necessary for normal lens to complete differentiation.

In summary, our present study has clearly demonstrated that SUMO1 and SUMO2/3 have distinct functions in regulating lens-cell differentiation, which is derived from their differential effects on Sp1 and possibly other transcription factors (Fig. 4*D*).

Methods

Mice used in this study were handled in compliance with the *Guide for the Care and Use of Laboratory Animals*. Embryonic and adult mice were obtained from the University of Nebraska Medical Center breeding facility.

Other analytical methods used in this study are detailed in *SI Methods*. Oligo primers used in the generation of various plasmids, qRT-PCR, and qChIP are listed in Tables S1 and S2.

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